Molecular Analysis of Bivalve Tumors: Models for Environmental/Genetic Interactions

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An increase in both the numbers and types of tumors found in finfish and shellfish has been noted in the past several decades. In many cases, while the increase in tumor incidence can be correlated with increases in aquatic toxicant levels, causality cannot be definitively proven. One recent epidemiologic investigation identified the prevalence of gonadal cancers as high as 40% in softshell clams (*Mya arenaria*) in Maine and 60% in hardshell clams (*Mercenaria spp.*) from Florida. A second study of these same geographic areas identified human mortality rates due to ovarian cancer as significantly greater than the national average. The rise in mortality rates in humans correlated with the increased use of herbicides in these areas as well as with the appearance of significant numbers of gonadal tumors in the clams. Studies were initiated in our laboratory to examine the molecular basis of these neoplasms in bivalves. NIH3T3 transfection assays were used to examine DNA isolated from these molluscan tumors for the presence of activated oncogenes. DNAs isolated from advanced tumors in both species were able to transform NIH3T3 cells and induce tumors in athymic mice. Studies are now underway to identify the gene(s) detected by these assays and also to examine the molecular mechanisms of toxic response of herbicide-exposed clams. — Environ Health Perspect 102(Suppl 12):81–83 (1994)

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Introduction

Recent ERL/N (U.S. EPA Environmental Research Laboratory, Narragansett) field surveys identified epizootic germinomas in three geographically distinct populations of softshell clams (*Mya arenaria*) in eastern Maine (*1*). Investigation into the etiology of these tumors revealed that all three locations had been subjected to herbicide exposure. Significant quantities of Tordon 101 (picloram), 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) had been used in forestry and in the culture of blueberries (*1*). 2,4-D and 2,4,5-T are not known to be potent carcinogens. However, TCDD (dioxin; 2,3,7,8-tetrachlorodibenzo-p-dioxin), a by-product contaminant from the synthesis of 2,4,5-T has been described as one of the most toxic environmental contaminants known (*2*). Histologically similar tumors were reported in hardshell clams (*Mercenaria* spp.) taken from the Indian River, Florida (*3*). This estuarine system drains a wide area of citrus groves and receives substantial domestic and agricultural runoff. The suggested association between herbicides and molluscan gonadal neoplasms was strengthened by critical laboratory studies of bivalves that linked rapid uptake of chemical carcinogens, mutagenic activity, and tumor formation (*4–6*).

Additional reports support the hypothesis that exposure to environmental toxicants may contribute to tumor induction. Bullhead catfish taken from lakes near Indian River exhibited a variety of neoplasms of epidermal origin (*7*). Seminomas have been reported in military working dogs exposed to herbicides in Vietnam (*8*). More recently, a positive correlation was found between the incidence of malignant lymphomas in dogs and their owners' use of phenoxyacetic acid herbicides on their lawns (*9*). Furthermore, an EPA survey of cancer mortality rates in the United States indicated that the mortality rate due to ovarian and other reproductive organ cancers in human females from Washington County, Maine, and near Indian River was significantly higher than the national average (*10*). These are the same geographic areas in which the tumor-bearing clam populations are located. Together, these similar toxicologic effects at different phylogenetic levels suggest that herbicides, and/or the contaminating TCDD, may contribute to tumor formation.

These data are correlative and suggest that more detailed studies are warranted. The degree to which exposure to environmental toxicants induces cancer has long been debated and in most cases, is impossible to prove. One approach is a comparative study of the basic underlying mechanisms of chemical carcinogenesis. Recent advances in molecular techniques have shown that many of these mechanisms are evolutionarily conserved (*11*).

To address this hypothesis, we initiated investigations of the molecular mechanisms of tumor formation correlated with environmental exposure to herbicides in the two bivalve species. We approached this problem from two directions. First, we examined DNA from clam tumors for the presence of activated oncogenes using DNA transfection. Our second approach was to address the possible molecular mechanisms of herbicide toxicity. Since the symptoms observed in polycyclic aryl hydrocarbon/dioxin-exposed animals seem to arise from the interaction of these compounds with the Ah receptor (*12*), we focussed on identifying and characterizing an analogous system in the clam.

DNA Transfection Analysis

Softshell clams (*M. arenaria*) and hardshell clams (*Mercenaria* spp.) were collected from Dennysville, Maine, and the Indian River, respectively. Tissue sections were fixed in Helly's fixative and the histopathologic analysis determined as previously described (*13*). Tissue for DNA extraction was rapidly frozen in liquid nitrogen and stored

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at –70°C. DNA was prepared by Quick-Dounce homogenization as reported earlier (*14*). DNAs from both clam species were co-transfected with the plasmid pSV_2neo into NIH3T3 cells using a calcium-phosphate precipitation procedure (*14,15*) modified from that of Graham and van der Eb (*16*). Stable transfectants were selected by growth in media containing G418 (Geneticin; Gibco/BRL). G418-selected cells were pooled and assessed for transformation/tumorigenicity in three assays: standard focus assay, colony selection assay and the nude mouse assay (*17*). Foci and colonies were picked and expanded. High molecular weight DNAs isolated from transformed cells were used to initiate a second transfection cycle as described previously (*15*). These data were confirmed by repeating the secondary transfection cycle, the results of which are presented in Table 1. DNA isolated from cells which were transfected with DNA from an advanced tumor in *Mercenaria* spp. and by both an early and an advanced tumor from *M. arenaria* were able to transform NIH3T3 cells in a secondary cycle of transfection. This confirms our studies reported previously (*15*). In addition, cells from the same pool (*M. arenaria* advanced tumor and *Mercenaria* spp. early tumor) were able to produce colonies in the colony selection assay when grown in the presence of low (0.1%) serum (data not shown). Only DNA from cells which were originally transfected with *M. arenaria* early tumor were able to induce tumors in nude mice within a short latent period (one of two mice at 6 weeks post-injection).

The results of our preliminary studies presented above indicate that clam tumor DNA is able to transform NIH3T3 cells. Although the identity of this gene(s) has not yet been determined, the data suggest that it is a highly conserved sequence since the gene is active in a mammalian transfection system. If our hypothesis is correct, that herbicide/dioxin exposure may be involved in formation of some gonadal tumors by a mechanism conserved in different species, then the identification of this gene would provide an important clue to this puzzle. Little is known about the molecular events in tumorigenesis in marine invertebrates. In women, however, ovarian carcinoma is one of the leading causes of death due to cancer (*18*) and its molecular basis is an active field of investigation. Activation of proto-oncogenes (K-*ras*, H-*ras*, c-*myc*, *HER-2/neu*) and mutated alleles of the *p53* tumor suppressor gene have been detected in ovarian tumors and ovarian tumor cell lines (*19–21*). The identification of the transforming genes in bivalves would enable us to compare their molecular mechanisms with those known in humans.

Table 1. Secondary transfection of NIH3T3 cells with clam tumor DNA.[a]

DNA source	Standard focus assay, no. foci/plate: Cells plated in DMEM	Cells plated in DMEM + Dex
Mya arenaria		
Reference animal	3	0
Early tumor	70	7
Advanced tumor	33	—
Mercenaria spp.		
Reference animal	20	1
Early tumor	0.5	—
Advanced tumor	90	6

Abbreviations: DMEM, Dulbecco's modified eagle medium; Dex, dexamethasone. [a]DNA isolated from primary transfectants was used in a second transfection cycle. Twenty micrograms of DNA were transfected per each plate. Cells in the standard focus assay were plated half in DMEM and half in DMEM with the addition of dexamethazone. Data from nude mouse experiments are discussed in the text. Results of the primary transfection assays and a previous secondary transfection cycle have been described earlier (*15*).

Identification of a Bivalve Ah Receptor

Studies are also underway to identify the bivalve analog of the Ah receptor and to define the role that it may play in these tumors. The Ah receptor has been identified in a number of vertebrate species including humans, rodents and trout (*22*). A number of genes which affect cellular growth and differentiation are in some way affected by interactions with the Ah receptor. Our preliminary results of photoaffinity ligand-binding assays (Brown *et al.*, unpublished) suggest that *Mercenaria* possess cytosolic proteins which specifically bind a dioxin analog (*23*). The presence of an Ah receptor complex in an invertebrate would suggest conservation over a long evolutionary time period and a critical role for the Ah receptor.

The outcome of these studies awaits further investigation. The role of herbicides in the etiology of these tumors will be examined in controlled laboratory exposures. We are continuing to investigate the role of suppressor genes and activated oncogenes in the bivalve gonadal tumors as well as the possible link to the transcriptional activation activity of the Ah receptor.

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